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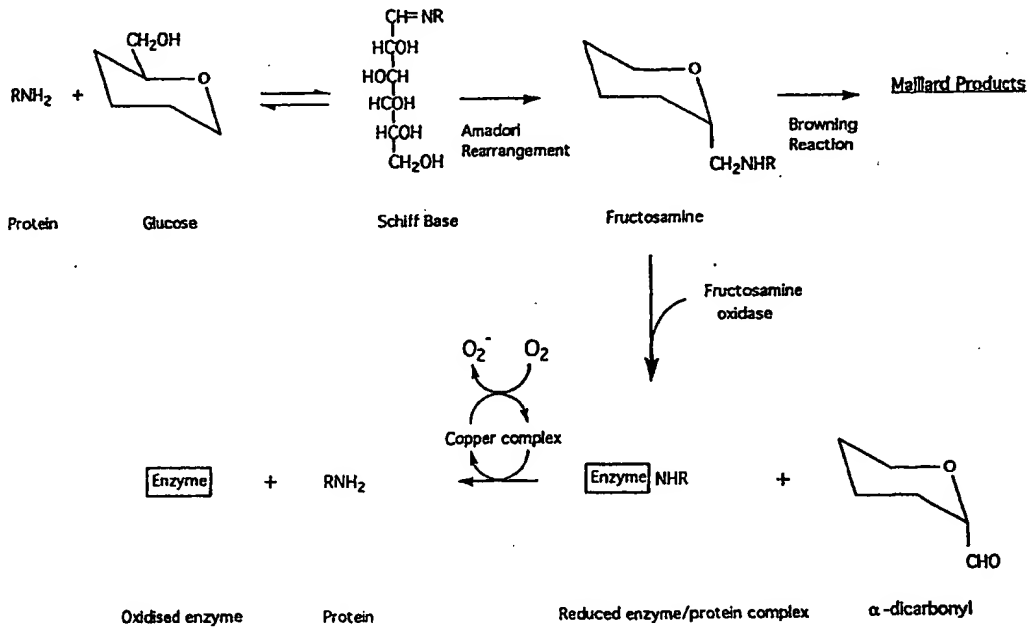

(57) Abstract

Methods of reducing the consequences of macrovascular and microvascular damage in a diabetic patient reliant upon the inhibition and/or antagonism of *Fructosamine oxidase* in the patient. Inhibition and/or antagonism can involve the use of copper chelating agents, substrate analogues or hydrazine compounds.

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"FRUCTOSAMINE OXIDASE: ANTAGONISTS AND INHIBITORS

THE CURRENT INVENTION

The present invention relates to methods of treatment, pharmaceutical compositions, dosage forms, uses of fructosamine oxidase enzyme inhibitors in medicine or for manufacturing pharmaceutical compositions, treatment regimes and related combinations, methods and products.

Diabetes mellitus is a common disorder affecting nearly 16 million Americans. See, Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care,* 20;1183-97 (1997). Diabetic individuals are prone to complications which are a major threat to both the quality and the quantity of life. Almost half those diagnosed with diabetes before the age of 31 years die before they reach 50 years largely as a result of cardiovascular or renal complications, often with many years of crippling and debilitating disease beforehand. See, Deckert T, Poulsen J, Larsen M. *Diabetologia* 14:363-70 (1978). It is estimated that diabetic individuals have a 25-fold increase in the risk of blindness, a 20-fold increase in the risk of renal failure, a 20-fold increase in the risk of amputation as a result of gangrene, and a 2- to 6-fold increased risk of coronary heart disease and ischaemic brain damage. See, Klein R, Klein B, Moss S, Davis M, DeMets D. *Diabetes Care* 8;311-5 (1985).

Largely because of these long-term complications, the cost of diabetes in the US was estimated as $98 billion in 1997 comprising $44 billion for direct medical costs such as inpatient and outpatient care plus $54 billion for indirect costs such as lost earnings and productivity, and premature death. Medical innovations that can slow the progression of diabetes have tremendous potential to mitigate the associated clinical and cost repercussions See, American Diabetes Association, "Economic consequences of diabetes in the US in 1997," *Diabetes Care* 21:296-309(1998).

Elevated blood glucose levels are now regarded as ***causative*** of diabetic complications based on results of the Diabetes Complications and Control Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS). See, *N Eng J Med.* 379:977-85 (1993) and *Lancet* 352:837-53 (1998). The DCCT and the

UKPDS have both demonstrated that the development of complications of diabetes are related with degree of hyperglycaemia and that long-term outcome may be ameliorated by rigorous treatment. For example, prognosis is dramatically improved if capillary blood glucose and glycated haemoglobin levels are maintained less than 150mg/dL and 7.0% respectively.

The mechanism of glucose toxicity in the tissues of patients with diabetes mellitus is unknown. Glucose condenses with free amino groups on structural and functional proteins to form Schiff bases which, in turn, undergo a series of transformations to yield dark-brown Maillard products. It has been proposed that diabetes complications are caused by the non-enzymatic cross-linking of proteins. See, Cerami A, Ulrich PC, Brownlee M, *US Patent* 4758583 (1988). However, although increased protein cross-linking is seen in the tissues of people long-standing diabetes, the role of Maillard products as a causative factor is certainly not clear. See, Wolff SP, Jiang ZY, Hunt JV. *Free Rad Biol Med* 10;339-52 (1991).

Amadori-rearrangement is the most important Maillard transformation because its product, fructosamine, is the precursor of all the browning products. ~~We~~ I have isolated a novel extracellular enzyme which catalyses the elimination of fructosamines from glycated protein. The existence of this enzyme has not previously been recognised in the world literature. Based on its high specificity for glycated protein substrates and its use of oxygen as acceptor, the enzyme may be classified as **fructosamine oxidase 1.5.3**. See, Enzyme Nomenclature, Recommendations of the Nomenclature Committee of the International Union of Biochemistry, Academic Press, London pp. 19-22, (1979).

Fructosamine oxidase is a metalloenzyme with copper & quinone cofactors and it belongs to the copper amine oxidase group of enzymes which have been isolated from bacteria, fungi, yeast, and mammalian sera. Products of the fructosamine oxidase catalysed reaction are free unglycated protein, α-dicarbonyl sugar, and the active oxygen species superoxide.

Increased fructosamine oxidase activity could cause many of the recognised sequelae of diabetes by degrading fructosamines bound to basement membrane proteins and generating reactive oxygen species as reaction products. For example,

superoxide anions cause an increase in intracellular calcium which modulates the activity of nitric oxide synthase. Nitric oxide is a potent vasodilator and it has been implicated in the vascular dysfunction of early diabetes. See, Ido Y, Kilo C, Williamson JR. *Nephrol Dial Transplant* 11 Suppl 5:72-5 (1996). Reactive oxygen species also cause a drastic dose-dependent decrease in *de novo* synthesis of heparan sulfate proteoglycans leading to a reduction in anionic sites on the glomerular basement membrane and an increase in basement membrane permeability to cationic plasma proteins such as albumin. See, Kashira N, Watanabe Y, Makin H, Wallner EI, & Kanwar YS. *Proc Natl Acad Sci USA* 89:6309-13 (1992). Increased urinary albumin clearance is a risk indicator in people with diabetes mellitus both for evolving renal disease and for early mortality mainly from coronary heart disease. See, Mattock MB, Barnes DJ, Viberti GC, et al. *Diabetes* 47:1786-92 (1998).

Once natural anti-oxidant defences are exceeded, hydroxyl radicals may be generated from superoxide via a copper catalysed Haber-Weiss reaction. See, Halliwell B & Gutteridge JMC "Free radicals in Biology and Medicine" Clarendon Press, Oxford pp. 136-76 (1989). Hydroxyl radicals are extremely reactive species and could cause the permanent site-specific damage to basement membrane proteins and histopathological changes that are typical of diabetic microvascular disease. See, Robbins SL, Cotran RS, Kumar V. "Pathologic basis of disease" 3rd ed WB Saunders, pp. 991-1061. (1984).

Similarly, any prolonged increase in fructosamine oxidase activity will cause oxidative stress which could account for the excess risk of macrovascular disease and the 75% increase in mortality seen in patients with diabetes mellitus compared with non-diabetic individuals. Recent studies have convincing demonstrated that oxidative modification of low density lipoprotein (LDL) is involved in the development of atherosclerosis of coronary and peripheral arterial vessels and elevated oxidised LDL concentrations are found in subjects with diabetes mellitus. See, Witztum JL *Br Heart J* 69 (Suppl):S12-S18 (1993) and Picard S, Talussot C, Serusclat A et. al. *Diabetes & Metabolism* 22:25-30 (1996). Oxidative changes to membrane lipids and to membrane protein SH-groups may also cause aberrations in cellular calcium homeostasis and contribute to the increased incidence of cardiac

sudden death that is typical of diabetes. See, Yucel D, Aydogdu S, Cehreli S et al. *Clin Chem* 44:148-54 (1998).

SUMMARY OF THE INVENTION

The existence of the fructosamine oxidase enzyme has not previously been recognised in the world literature. This is a novel enzyme. I believe that excess fructosamine oxidase activity with glycated basement membrane protein substrate plays a vital role in diabetic complications by the formation of α-dicarbonyl and reactive oxygen free radical species.

I also believe that this damage may be ameliorated by administering specific fructosamine oxidase inhibitors or antagonists selected from the groups: (i) copper chelating agents; (ii) substrate analogues; & (iii) hydrazine compounds.

As used herein (including in the claims) the terms -

- "copper chelating agents" means any agent which reduces body fructosamine oxidase activity (e.g. by depleting body copper stores or by binding and inactivating the copper molecule at the reactive centre of the enzyme) which is capable of being administered in effective amounts by any appropriate administration route (eg. orally, by injection etc). See some examples hereafter referred to.
- "substrate analogue" means any chemically modified amino acid or peptide substrate which inactivates fructosamine oxidase (e.g. by binding (preferably substantially) irreversibly to the reactive centre of the enzyme) which is capable of being administered in effective amounts by any appropriate administration route (eg. orally, by injection etc). See some examples hereafter referred to.
- "hydrazine compound" means any agent containing the moiety -NH-NH_2 which inactivates fructosamine oxidase (e.g. by binding and inactivating the quinone molecule at the reactive centre of the enzyme) which is capable of being administered in effective amounts by any appropriate administration route (eg. orally, by injection etc). See some examples hereafter referred to.
- "at least periodically" includes from a single administration to continuous

administration.

- "macrovascular and microvasular damage" refers to both general types of damage but can refer to one general type of such damage provided there is a view to minimising or ameliorating the consequences of and/or likelihood of such category of damage.
- "comprises" can mean "includes".
- "and/or" means both "and" and "or".
- "in concert with" does not necessarily mean as a result of simultaneous administration or self administration (eg; can be serially and such serial application can be spaced, ie; triene between meals and another agent with a meal).
- "triethylenetetramine dihydrochloride" or "triene" includes for the target mammalian species or for a human being any pharmaceutically acceptable fructosamine oxidase enzyme inhibiting and/or antagonising analogue or metabolite thereof (eg; an acetylated derivative) capable of administration or self administration in an amount alone, or in concert with another fructosamine oxidase enzyme inhibitor and/or antagonist (preferably not contraindicated by toxicity concerns having regard to levels required for effective inhibition and/or antagonism), of providing effective inhibition and/or antagonism.

In one aspect the present invention consists in a method of treating a mammalian patient (human or otherwise) predisposed to and/or suffering from diabetes mellitus with a view to minimising the consequences of macrovascular and microvascular damage to the patient (eg. accelerated atherosclerosis, blindness, renal failure, neuropathy, etc.) which comprises, in addition to any treatment in order to control blood glucose levels, at least periodically inhibiting or antagonising fructosamine oxidase enzyme activity in the patient.

Preferably said inhibition or antagonism occurs as a result of administration or self-administration of at least one fructosamine oxidase reaction product inhibitor or antagonist.

Preferably any such inhibitor or antagonist is selected from the groups

(i) copper chelating agents

(ii) substrate analogue

(iii) hydrazine compound

Preferably said inhibitor or antagonist is taken orally.

Preferably said inhibitor or antagonist is taken orally as part of a regime, whether totally oral or not, which also involves the control of blood glucose levels.

In a further aspect the present invention consists in a pharmaceutical composition (preferably oral) suitable for use in such a method, said composition comprising a fructosamine oxidase inhibitor or antagonist in conjunction with a suitable carrier therefor.

In yet a further aspect the present invention consists in a pharmaceutical composition for reducing macrovascular and microvascular damage in a mammalian patient (including a human) suffering from diabetes mellitus, said composition comprising a fructosamine oxidase inhibitor or antagonist and suitable carrier therefor.

Preferably said carrier can be any diluent, excipient or the like and the dosage form of said pharmaceutical composition can be of any appropriate type whether for oral or other administration or self administration. Long acting release forms are also envisaged within the present invention.

In still a further aspect the present invention consists in the use of a fructosamine oxidase inhibitor or antagonist in the manufacture of a pharmaceutical composition comprising the fructosamine oxidase inhibitor or antagonist and a suitable pharmaceutical carrier therefor and which composition is useful in treating a mammalian patient (human or otherwise) which or who is suffering from diabetes mellitus to reduce macrovascular and microvascular damage (preferably by a method of the present invention).

In still a further aspect the present invention consists in combination, the treatment regimes and/or the medicaments of such regimes previously set forth whether packed together or prescribed together or otherwise.

In still another aspect the invention consists in a method of treating a mammalian patient (human or otherwise) predisposed to and/or suffering from

diabetes mellitus, which includes inhibiting or antagonising fructosamine oxidase enzyme activity in the patient with an agent or agents preferably not contraindicated for the patient. Examples of inhibitor or antagonist include but are not limited to those listed hereinafter.

Preferably in one embodiment said agent(s) is or are copper chelating compound(s) administered or self administered to the patient.

Examples of suitable copper-chelating compounds include triethylenetetramine dihydrochloride (triene), penicillamine, sar, diamsar, ethylenediamine tetraacetic acid, *o*-phenanthroline, & histidine.

Preferably in another embodiment said agent(s) is or are substrate analogue compound(s) administered or self administered to the patient having an amino acid or peptide moiety with a blocked N-terminal amine group.

Examples of a suitable substrate analogue composition is *N*-acetylcysteine, captopril, lisinopril & enalapril).

Preferably in another embodiment said agent(s) is or are hydrazine compound(s) administered or self administered to the patient ie: a compound having a -$NHNH_2$ moiety.

Examples of a suitable hydrazine compound include diaminoguanidine, hydralazine, & carbidopa.

In still another aspect, the invention consists in a dosage regimen for a method of the present invention and/or using dosage units of the present invention

In still a further aspect, the present invention consists in the use of a pharmaceutical acceptable compounds being at least one of a substrate analogue, a hydrazine compound and a copper chelator in the manufacture of a dosage unit or pharmaceutical composition useful in treating a patient (human or otherwise) which or who is suffering from diabetes mellitus to reduce macrovascular and microvascular damage.

In another aspect, the invention consists in a dosage unit or pharmaceutical composition for a patient useful in a method of the present invention comprising (preferably in effective fructosamine oxidase reaction product inhibiting or antagonising amounts - separately or collectively) of

a compound (or compounds) being a substrate analogue or a hydrazine compound having an -$NHNH_2$ moiety, or both.

Preferably said dosage unit also includes or said pharmaceutical composition also includes a compound (or compounds) (preferably different compound(s)) which is (or are) a copper chelator (or copper chelators).

Preferably said dosage unit or composition is in an oral dosage form optionally with carriers, excipients or, indeed, even other active agents (e.g. means to lower blood glucose levels).

In still another aspect the invention consists in a regime or dosage unit or pharmaceutical composition for a diabetic or suspected diabetic patient of the copper chelator, triene, providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

In still another aspect the invention consists in a regime or dosage unit or pharmaceutical composition for a diabetic or suspected diabetic patient of captopril [whether effective or intended to be effective in controlling blood pressure of the diabetic patient (at least in part) or not] providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

In yet another aspect the invention consists in a regime or dosage unit or pharmaceutical composition for a diabetic patient or suspected diabetic patient of a hydrazine compound providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

In yet another aspect the invention consists in a regime or dosage unit or pharmaceutical composition for a diabetic patient or suspected diabetic patient of

(i) acetylcysteine and

(ii) at lease one other fructosamine oxidase inhibitor and/or antagonist, the mix of (i) and (ii) providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

In yet another aspect the invention consists in a regime or dosage unit or

pharmaceutical composition for a diabetic patient or suspected diabetic patient of

(i) hydralazine and

(ii) at lease one other fructosamine oxidase inhibitor and/or antagonist, the mix of (i) and (ii) providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

In still another aspect the present invention consists in a method of treating a mammalian patient (human or otherwise) predisposed to and/or suffering from diabetes mellitus which includes inhibiting and/or antagonising fructosamine oxidase enzyme activity in the patient with acetycysteine and hydralazine.

In still another aspect the invention consists in a regime or dosage unit or pharmaceutical composition for a diabetic or suspected diabetic patient which includes acetylcysteine and hydralazine.

In still a further aspect the present invention consists in the use of co-administration or serial administration of acetylcysteine and hydralazine for the purpose of reducing macrovascular and microvascular damage in a mammal.

Preferably said mammal is diabetic.

In yet another aspect the invention consists in a method of treating and/or reducing the likelihood of diabetic cataract in a mammal which comprises at least periodically inhibiting and/or antagonising fructosamine oxidase enzyme activity in the mammal.

Preferably the method involves the administration or self administration of effective amounts of triethylenetetramine dihydrochloride (triene).

In another aspect the invention consists in a method of treating and/or reducing the likelihood of diabetic cardiomyopathy in a mammal which comprises at least periodically inhibiting and/or antagonising fructosamine oxidase enzyme activity in the mammal.

Preferably the method involves the administration or self administration of effective amounts of triethylenetetramine dihydrochloride (triene).

Preferably for any of the aforesaid indications triethylenetetramine dihydrochloride (triene) is administered and/or self administered in concert with

another (other) fructosamine oxidase enzyme inhibitor(s) and/or antagonist(s).

Preferably said another inhibitor and/or antagonist or said other inhibitors and/or antagonists is or are administered or self administered to elicit a pharmacological effect for another indication yet together with the effect of the triene is or are in an amount or amounts which are effective for treating or ameliorating macrovascular and microvascular damage of such a patient or mammal.

Reference is drawn to my PCT Application filed simultaneously herewith (claiming priority of New Zealand Patent Specification No. 332085 filed 25 September 1998), the full content of which is hereby included by way of reference. It discloses methods of monitoring fructosamine oxidase inhibition and/or antagonism of patients, screening and/or determine patients to determine patients at risk to vascular (particularly microvascular) damage and identifying those individuals who will benefit by treatment with fructosamine oxidase inhibitors and/or antagonists, methods of determining fructosamine oxidase levels in a mammal, methods of determining blood plasma fructosamine oxidase levels in a diabetic individual or a suspected individual, methods of assaying blood serum or blood plasma *in vitro* for fructosamine oxidase, methods of identifying or testing candidate substances and to related methods and procedures.

Preferably the measurement conducted *in vitro* is of the superoxide reaction product (or any other oxygen free radical product) of fructosamine oxidase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a detailed reaction mechanism for the formation of fructosamine and Maillard products from glucose and protein. Fructosamine oxidase degrades fructosamine by a two-step reaction with initial release of an α-dicarbonyl sugar and subsequent oxidation of the enzyme/protein complex to release free unglycated protein. The reduced copper cofactor is oxidised *in vivo* by molecular oxygen and the oxidation product is superoxide.

Figure 2 shows absorbance spectra of the fructosamine oxidase enzymes extracted from pooled human sera (A) and from the microbial organism, *Enterbacter aerogenes* (B).

Figure 3 shows spectra of *p*-nitrophenylhydrazine (NPH) adduct of the *Enterbacter aerogenes* enzyme (A) and a red absorbance shift when the NPH-enzyme adduct is diluted in 2M KOH.

Figure 4 shows survival curve for non-treated STZ-diabetic rats compared with diabetic animals treated with fructosamine oxidase inhibitors.

Figure 5 shows monthly growth of treated and untreated STZ-diabetic rats compared with non-diabetic animals.

DETAILED DESCRIPTION OF THE INVENTION

(i) Extraction of holoenzyme

Fructosamine oxidase in blood plasma is largely found as an enzyme-substrate conjugate, bound to peptides and proteins (Fig 1). To obtain a maximal yield of active holenzyme, it is necessary to make the pH of the media alkaline preferably with phosphate buffer, to add sulphydryl reagents, and to incubate the mixture with pro-oxidant so that glycated species are released. Most effective activation is found with cupric salts.

Fructosamine oxidase holenzyme is separated from inactive apoenzyme by affinity adsorption chromatography. A suitable glycated affinity support is prepared from alkylamine beads or beaded cross-linked agarose with amino terminal residues attached by 6-10 atom spacer arms (available from Pierce™, Bio-Rad™, & Pharmacia™). Affinity support is glycated by incubating with 400mM potassium phosphate buffer pH 7.4 containing 50mM glucose and 0.01% sodium azide at 37 °C for 7 days. Holoenzyme binds tightly to glycated amino residues and residual copper is readily removed by washing with water. Active holoenzyme is eluted with 800mM NaCl in 50mM sodium acetate buffer pH 4.8. Active fractions are pooled and protein is precipitated with 50% cold acetone solvent. The protein pellet is reconstituted with a minimal volume of water or physiological saline and lyophilised for long term storage.

Extraction of 35mL pooled diabetic and non-diabetic human sera yielded a clear colourless preparation with absorbance peaks at 196nm & 264nm typical of the absorbance spectra of fructosamine oxidase (Fig 2). A fructosamine oxidase enzyme

from *Enterobacter aerogenes* showing absorbance peaks at 196nm & 255nm is included for comparison. Enzyme activity and relative activity is as follows.

TABLE 1.

Sample	Protein (µg/mL)	Cytochrome c activity* (U/L)	Sp activity† (U/g)
human	32.9	4.58	139.4
E aerogenes	541.5	66.32	115.11

* Enzyme extract was preincubated in 0.05M TES buffer pH 7.4 containing 1mM DMF substrate at 37 °C for 5 minutes. Enzyme activity was measured with 10µM ferricytochrome c. The reaction was started with 50µM fructosamine substrate as g-BSA and ΔA_{550nm} was determined over 5 minutes.

† Protein concentration determined from A_{210nm}-A_{220nm} compared with BSA standards.

(ii) Cofactor identification

The *p*-nitrophenylhydrazine (NPH) adduct of *Enterbacter aerogenes* enzyme with A_{max} 399nm was obtained as described previously. See, Palcic MM, Janes SM. *Meth Enzymol* 258:34-8 (1995). A red absorbance shift to A_{max} 438nm was observed when the NPH-enzyme adduct was diluted in 2M KOH. Such an absorbance shift is typical of the quinone cofactors of copper amine oxidase.

EXAMPLE 1: Identifying fructosamine oxidase inhibitors

The purpose of this example is to demonstrate how the fructosamine oxidase assay, the subject of a simultaneously filed PCT International patent specification NZ 332085, may be used in identifying and grading candidate fructosamine oxidase inhibitors. This approach takes into account the activity of the drug in a human plasma matrix *in vitro*. Enzyme inhibitors find wide application in clinical medicine as treatments for a range of metabolic disorders. For example, angiotensin converting enzyme inhibitors have been used in the treatment of hypertension. See, Harris EE, Patchett AA, Tristram EW, & Wyvratt MJ. Aminoacid derivatives as antihypertensives. US Patent 4374829 (1983). Similarly, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase enzyme inhibitors have been used in the treatment of hypercholesterolaemia. See, Hoffman WF, Smith RL, Lee

TJ. Novel HMG-CoA reductase inhibitors. US Patent 4866090 (1989). *Fructosamine oxidase* inhibitors may be selected from those substances which bind and block the quinone co-factor (hydrazine compounds), the copper co-factor (copper chelators), or which mimic the normal substrate of the enzyme (substrate analogue).

Method:

Potential *fructosamine oxidase* inhibitors were tested on human serum or plasma (individually and in combination) using the method of assaying *fructosamine oxidase* activity described in detail in a New Zealand Patent Specification No. 332085. Irreversible enzyme inhibition is characterised by a progressive decrease in activity with time ultimately reaching complete inhibition even with very dilute inhibitor concentrations provided that the inhibitor is in excess of the amount of enzyme present.

Results:

The relative activity of a selection of hydrazine, copper chelator, and substrate analogue *Fructosamine oxidase* inhibitors are shown in TABLE 2. In some instances, there was a degree of overlap between classes i.e. some hydrazine compounds are also copper chelators. To clarify this point, copper chelating potential for some compounds is indicated (β). The effectiveness of the inhibitor is expressed not by an equilibrium constant but by a velocity constant (K) which determines the fraction of the enzyme inhibited in a given period of time by a certain concentration of inhibitor. The specificity of the inhibitor for the active centre of the enzyme is indicated by the concentration of inhibitor CAUSING 50% inactivation of the enzyme (IC_{50}).

TABLE 2

Inhibitor: Hydrazine compounds	IC_{50}[1]	$K(min^{-1})$[2]	β[3]
aminoguanidine	231.2μM	0.0067*	-
semicarbazide	45.1μM	0.0276*	+++
benserazide	13.6μM	0.0095*	
oxalic dihydrazide	1.59μM	0.0542	-
hydralazine	1.52μM	0.0029	+++
phenylhydrazine	0.81μM	0.1160	-
carbidopa	0.50μM	0.1496	
diaminoguanidine	0.36μM	0.1340	-
Inhibitor: Substrate analogues			
lisinopril	216.9μM	0.0174	
enalapril	3.95μM	0.0326	+++
captopril	1.78μM	0.0259	-
acetylpenicillamine	1.06μM	0.0811	
acetylcysteine	0.83μM	0.1677	
Inhibitor: Copper chelators			
desferrioxamine	40.6μM	0.0109*	
EDTA	15.7μM	0.0755*	
Sodium azide	9.48μM	0.0004	
Potassium cyanide	6.36μM	0.0116	
triene	5.40μM	0.0196	
o-phenanthroline	4.25μM	0.0385	
histidine	2.29μM	0.0554	
Inhibitor: Combined agents			
acetylcysteine + hydralazine	0.57μM	0.1654	
acetylcysteine + diaminoguanidine	1.07μM	0.0795	
acetylcysteine + histidine	1.11μM	0.0722	
acetylcysteine + carbidopa	0.27μM	0.2000	

1 fresh human sera was incubated with 0-1,000μM inhibitor in 0.05M TES buffer pH 7.4 at 37 °C for 5 minutes. Enzyme activity was measured with 10μM ferricytochrome c. The reaction was started with 50μM fructosamine substrate as g-BSA and ΔA_{550nm} was determined over 5 minutes.

2 rate constants were calculated from the reaction of fructosamine oxidase either with 1.0μM inhibitor or with 10.0μM inhibitor (*).

3 copper chelating potential (β) was determined from ability of agent to remove copper under dialysis from copper-saturated BSA substrate.

CONCLUSION:

1. Irreversible inhibition of *fructosamine oxidase* is feasible.
2. Inhibitors may be broadly categorised in three classes of compound: hydrazines; substrate analogues; & copper chelators.
3. *Fructosamine oxidase* activity in human blood plasma may be eliminated by micromolar concentrations of inhibitors.
4. Many of the active inhibitors are drugs which have already been administered as medicines in humans to treat other disorders (not diabetes).

EXAMPLE 2: Clinical Utility of *fructosamine oxidase* inhibition: First Preclinical study

The purpose of this example is to demonstrate how the clinical usefulness of candidate *fructosamine oxidase* inhibitors may be assessed using a standard animal model of diabetes mellitus, the streptozocin-diabetic rat (STZ rat). This approach takes into account drug bioavailability, the activity of the drug and its metabolites, and any drug adverse effects or toxicity factors.

Method:

48 WISTAR rats aged 6-8 weeks & weighing 200-300g were randomized:

- Group 1 Non-diabetic control
- Group 2 Diabetic control
- Group 3 Diabetic treated with hydralazine
- Group 4 Diabetic treated with EDTA
- Group 5 Diabetic treated with hydralazine & acetylcysteine
- Group 6 Diabetic treated with acetylcysteine.

Streptozotocin (60mg per kg) was administered into a lateral tail vein. Non-diabetic controls received a sham injection of buffer. Diabetes was confirmed by venous blood glucose measurement >15mmol/L after 1 week & diabetic animals were treated with subcutaneous injections of ultralente insulin (4U/injection) 3-5 days per week to maintain body growth. Medications were administered 50mg/L in

the drinking water over an 8 month period. Timed urine collects and venous plasma samples were obtained at monthly intervals.

Results:

(a) Blood glucose control: Rate of conversion to diabetes with intravenous STZ administration was >95%. Intravenous STZ induced a severe form of insulin-dependent diabetes which was sustained over the entire 8 month duration of the study. Despite insulin replacement therapy, glycaemia control was poor as evidenced by mean ± SD glucose (week 4) and HbA_{1c} (week 32) levels in TABLE 3.

TABLE 3.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Glucose (mmol/L)	9.1±1.5	30.1±9.7	35.7±9.5	39.0±6.4	30.4±8.8	37.8±5.2
HbA_{1c} (%)	3.92±0.11	10.85±0.05	8.65±1.18	9.30±0.63	8.72±0.55	9.47±1.23

(b) Survival: Mortality rate amongst untreated STZ rats was extremely high. Survival was improved significantly by the administration of *fructosamine oxidase* inhibitors (TABLE 4).

TABLE 4.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Survivors at week 32	8	2	6	5	8	7
Significance*	-	-	ns	ns	P <0.025	P <0.05

* Chi-square test compared with untreated STZ rats (Group 2)

The survival curve for STZ rats compared with non-diabetic controls is shown in FIGURE 4. Death was presumed secondary to a cardiovascular event. In general, renal function remained normal.

(c) Weight gain: There was a progressive weight gain amongst non-diabetic controls over the 32 weeks of the study which was abolished in the STZ diabetic animals. At the end of the 32 week study period, mean weight change amongst

surviving study animals was: Group 1, +74.6%; Group 2, -21.0%, Group 3, -11.0%%; Group 4, +1.2%, Group 5, +16.0%; & Group 6, -8.1% (FIGURE 5). Compared with untreated diabetic controls, fructosamine *oxidase* inhibitors caused an improvement in weight gain roughly in proportion to the activity of the inhibitor (TABLE 2) i.e. acetylcysteine/hydralazine > EDTA > acetylcysteine > hydralazine.

(d) Clinical pharmacokinetics:

- **Hydralazine** The bioavailability of hydralazine in man after oral administration is 26-55%. However, only 2.0-3.6% of the drug is excreted in the urine unchanged over 24 hours after oral administration. Most of the drug is recovered as an inactive acetylated product. See, Talseth T, *Eur J Clin Pharmacol* 10:395-401 (1976) and Talseth T, *Clin Pharmacol Ther* 21:715-20 (1977). This could account for the reduced efficacy of hydralazine as a *fructosamine oxidase* inhibitor in the current study. Furthermore, drug doses administered to each STZ rat were calculated as 12.5mg hydralazine/day or 35mg/kg, based on an average consumption 250mL water per day and assuming a mean body mass 350g. This rat dose far exceeds the maximum recommended human dose of 200mg hydralazine per day (3mg/kg assuming a mean body mass 70kg).
- **EDTA** The bioavailability of EDTA after oral administration is very low (less than 5%) because of poor absorption from the gut limiting its usefulness in humans to parenteral administration or irrigation techniques. See, Wynn JE et al *Toxicol Appl Pharmacol* 16:807-17 (1970).
- **Acetylcysteine** Acetylcysteine is rapidly absorbed from the gut with an bioavailability in man varying between 6 and 10%. See, Borgstrom L et al *Eur J Clin Pharmacol* 31:217-22 (1986). However, the drug is rapidly degraded in the liver by elimination of the acetyl moiety. See, Holdiness MR. *Clin Pharmacokinet* 20:123-34 (1991). Induction of liver enzymes could account for the progressive loss of drug efficacy seen after week 12 in the current study.

CONCLUSION:

5. Streptozocin induces a severe form of insulin dependent diabetes in the rat with a high morbidity and mortality.
6. Survival of STZ rats was enhanced by treating with *fructosamine oxidase* inhibitors in proportion to their activity in an *in vitro* assay.
7. Weight gain of STZ rats was enhanced by treating with *fructosamine oxidase* inhibitors.
8. There was benefit in co-administering acetylcysteine and hydralazine suggesting a synergy effect between classes of *fructosamine oxidase* inhibitors.
9. Based on these *in vivo* studies in the rat, the efficacy of a candidate of *fructosamine oxidase* inhibitor in a human is likely to be influenced by bioavailablity of the drug, degradation of the active compound *in vivo*, and maximum oral tolerated dose of the drug.

EXAMPLE 3: Clinical utility of *fructosamine oxidase* inhibition: Second Preclinical study

The purpose of this example is to demonstrate how the clinical usefulness of candidate *fructosamine oxidase* inhibitors, alone and in combination, may be assessed using a standard animal model of diabetes mellitus, the streptozocin-diabetic rat (STZ rat). This approach takes into account drug bioavailability, the activity of the drugs and their metabolites, interactions between drugs, and any drug adverse effects or toxicity factors.

Method:

80 Wistar rats weighing 200-300g and aged of 6-8 weeks were randomized:

Group 1	Non-diabetic control
Group 2	Diabetic control
Group 3	Diabetic treated with captopril (substrate analogue)
Group 4	Diabetic treated with carbidopa (hydrazine)
Group 5	Diabetic treated with triene (copper chelator)

Group 6 Diabetic treated with captopril & triene.

Group 7 Diabetic treated with captopril & carbidopa

Group 8 Diabetic treated with triene & cardidopa.

Diabetes was induced by administering streptozotocin (60mg per kg) by intraperitoneal injection. Non-diabetic controls received a sham injection of buffer. Diabetes was confirmed by venous blood glucose measurement >15mmol/L after 1 week & diabetic animals were treated with subcutaneous injections of ultralente insulin (4U/injection) 3 days per week to maintain body growth. Medications were administered 50mg/L in the drinking water over an 6 month period. Timed urine collects and venous plasma samples were obtained at monthly intervals. Animals were sacrificed and subjected to post-mortem at the end of the study.

Results:

(a) Blood glucose control: Rate of conversion to diabetes with intraperitoneal STZ administration was ≈80%. Poor glycaemic control was sustained over the 6 month duration of the study as evidenced by mean ± SD HbA_{1c} (week 4, 12, & 24) levels (TABLE 5).

TABLE 5.

HbA_{1c} (%)	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Week 4	4.1±0.1	8.3±0.9	8.5±0.9	9.0±1.0	8.0±1.0	9.0±5.2	9.1±1.5	9.1±1.5
Week 12	4.1±0.1	9.2±0.6	9.2±1.1	9.6±0.7	8.8±0.9	9.5±0.8	9.5±1.0	9.3±0.9
Week 24	3.7±0.1	9.4±1.3	9.6±1.3	9.9±1.1	9.0±1.4	9.5±1.3	9.8±1.2	9.1±1.2

(b) Survival: Compared with intravenous administration of STZ, intraperitoneal adminstration of STZ induced a less severe form of diabetes with lesser mortaility rate. At the end of the 24 week study period, mortality rate amongst study animals was: Group 1, 0%; Group 2, 14.3%, Group 3, 0%; Group 4, 0%, Group 5, 0%; Group 6, 12.5%, Group 7, 0%, & Group 6, 0%. There was no significant difference between groups because of the low frequency of events.

(c) Weight gain: STZ diabetes causes a profound weight loss in diabetic rats compared with non-diabetic controls. Mean weight gain of study animals from the

beginning to the end of the 24 week period are indicated in TABLE 6.

TABLE 6.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Mean ± SEM weight gain	342.8 ± 13.7	54.4 ± 12.5	60.7 ± 20.7	33.7 ± 20.4	123.6 ± 20.5	56.1 ± 21.3	55.1 ± 17.1	75.8 ± 25.4
P *	-	ns	ns	ns	0.0138	ns	ns	ns

- Student's t test compared with untreated STZ rats (Group 2)

In terms of general well-being, triene (Group 5) appears more effective than captopril (Group 3) and carbidopa (Group 4). The usefulness of triene was reduced when the drug was co-administered with captopril (Group 6) or carbidopa (Group 8). There is no evidence of synergy between classes of *fructosamine oxidase* inhibitors.

(d) Cataract formation Cataract is a recognised long-term complication of poorly controlled diabetes. Gross cataract formation in STZ rats compared with diabetic control animals by the end of the study at week 24 is shown (TABLE 7) .

TABLE 7.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
No (%) with cataract	0 (0%)	8 (40%)	2 (25%)	2(25%)	0 (0%)	2(28%)	5(62%)	1(12%)
P	-	-	ns	ns	< 0.10	ns	ns	ns

- Chi-square test compared with diabetic control rats (Group 2)

Although not significant at the P = 0.05 level, triene appears more effective than captopril and carbidopa in inhibiting gross cataract formation. There is no evidence of synergy between classes of *fructosamine oxidase* inhibitors.

(e) Diabetic cardiomyopathy Cardiomyopathy is a recognised long-term complication of poorly controlled diabetes. Macroscopically, hearts of STZ rats were dilated with thinning of the ventricular wall. Sections stained with haematoxylin and eosin and Masson's Trichrome showed focal pallor with a loss of normal

architecture in the myocardium of both ventricles that began at the sub-endocardial and sub-epicardial regions and spread to encompass the whole ventricular wall in severely affected animals. There was also marked infiltration by fibrous connective tissue between myocytes and increased fibrous connective tissue in the walls of intramural arteries. These appearance are consistent with dilated cardiomyopathy. Gross myocardial fibrosis in STZ rats compared with non-diabetic control animals by the end of the study at week 24 is shown (TABLE 8) .

TABLE 8.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
No (%) rats with severe fibrosis	0 (0%)	10 (50%)	6 (75%)	2 (25%)	0 (0%)	6 (75%)	8 (100%)	7 (87%)
P	-	-	ns	ns	< 0.005	ns	<0.005	<0.05

• Chi-square test compared with diabetic control rats (Group 2)

Triene appears highly effective in inhibiting the development of diabetic cardiomyopathy.. There is no evidence of synergy between classes of *fructosamine oxidase* inhibitors.

(f) Clinical pharmacokinetics:

- **Triene** The bioavailability of triene is less than 10%. Most of the unchanged drug is cleared in the urine within the first 6 hours of oral dosing mainly as an acetyl derivative indicating that a three or four times daily drug regimen or a sustained release preparation will be required. See, Kodama H et al *Life Sci* 61:899-907 (1997). In addition, plasma levels in non-fasted rats are significantly lower than those observed in fasted animals and the uptake of triene from the intestinal brush border is competitively inhibited by other amine compounds. See, Tanabe R et al *J Pharm Pharmacol* 48:517-21 (1996). This implies that triene is best administered in the fasting state. Interference in the absorption of drug from the intestinal brush border could account for

discrepancies between triene treatment groups (Groups 5, 6, & 8). Finally, in the current study, each STZ rat consumed approximately 250mL water per day (12.5mg triene/rat/day). Assuming a mean body mass 350g, this dose of triene equates to 35mg/kg. The dose of triene previously used in treating humans with another condition (not diabetes) ranges 1.2-2.4g (17-35mg/kg assuming a mean body mass 70kg). See, Walshe JM *Lancet* 8273:643-7 (1982). This implies that humans may be safely treated with comparable doses of trienes to those administered to rats in the current study to thereby elicit the fructosamine oxidase inhibition and/or antagonism advantages in a diabetic patient referred to herein.

- **Captopril** The bioavailability of captopril is approximately 65% after an oral dose. However, the drug is almost completely bound *in vivo* to albumin and other plasma proteins, and forms inactive mixed disulphides with endogenous thiols so that plasma levels of active drug may be very low. The elimination half life of unchanged captopril is approximately 2 hours. See, Duchin KL et al *Clin Pharmacokinet* 14:241-59 (1988). These observations might explain the reduced efficacy of captopril in the STZ rat compared with *in vitro* studies. Furthermore, each STZ rat consumed approximately 12.5mg captopril/day which equates to 35mg/kg assuming a mean body mass 350g. This dose far exceeds the maximum recommended human dose of 150mg captopril per day (2mg/kg assuming a mean body mass 70kg).
- **Carbidopa** In a study of beagle dogs, the oral absorption of carbidopa was almost complete and the absolute bioavailability was 88%. The biological half-life was 5 hours. See, Obach R et al *J Pharm Pharmacol* 36:415-6 (1984). However, carbidopa is an unstable compound and it degrades naturally in a short period. Solutions left to stand exposed to light at room temperature will undergo 50% oxidative degradation in 24 hours. See, Pappert EJ et al *Movement Disorders* 12:608-23 (1997). Reduced bioavailability due to oxidative degradation of the active drug both prior to its consumption and post-ingestion in the rat could explain (in part) the reduced efficacy of carbidopa in the current study. Finally, each STZ rat consumed approximately 12.5mg

carbidopa/day which equates to 35mg/kg assuming a mean body mass 350g. This dose far exceeds the maximum recommended human dose of 200mg carbidopa per day (3mg/kg assuming a mean body mass 70kg).

CONCLUSION:

10. Intraperitoneal streptozocin is associated with a lower mortality rate than intravenous streptozocin in the rat.
11. Weight gain was enhanced in STZ rats treated with the copper chelator, triene. Captopril and carbidopa were ineffective.
12. Cataract development may be inhibited by triene. Efficacy of triene is diminished when the drug is co-administered with either captopril or carbidopa.
13. The development of diabetic cardiomyopathy was prevented by treatment with triene. Efficacy of triene is diminished when the drug is co-administered with either captopril or carbidopa.
14. Oral doses of triene which inhibit the development of complications in the rat (cataract, cardiomyopathy, and early death) are equivalent on a body mass basis to doses of triene which have previously been used to treat human beings with another condition (not diabetes).
15. When administered to humans on a three or four times daily basis or as a sustained release preparation in previously tolerated doses 1.2-2.4g/day, triene may provide an effective means of treating the long-term complications of diabetes mellitus.

EXAMPLE 4: Clinical Utility of *fructosamine oxidase* inhibition: double-blind, placebo-controlled clinical trial

The purpose of this example is to demonstrate how the clinical usefulness of candidate *fructosamine oxidase* inhibitors will be assessed in diabetic human subjects. A detailed protocol based on this proposal has been approved by the Auckland Regional Ethics Committee. This approach takes into account drug bioavailability, the activity of the drugs and their metabolites, interactions between

drugs, any drug adverse effects or toxicity factors and the "scale-up" factor from rat to human treatment..

Objective: A pilot study to determine whether triene will reduce the rate of progression of renal disease and associated microvascular complications in patients with diabetic nephropathy due to NIDDM.

Patient population: 60 men and women aged between 40 years and 70 years of age with poor blood glucose control and diabetic nephropathy due to NIDDM.

Study design and duration: Randomised double-blind, placebo-controlled study design consisting of five periods:

- screening period (detecting possible candidates who meet study criteria);
- enrolment period (securing informed consent & baseline measurements);
- run-in period (trial of acceptability of study protocols & study medication);
- maintenance period (treatment with drug/placebo, monitoring efficacy/safety);
- follow-up period (detect any untoward effect when medication is disconitinued).

Blinded therapy (triene 400mg or placebo) will be administered three time daily ½ hour before meals in addition to current antihypertensive and hypoglycaemic therapies. The study will terminate when all patients are randomised and have been in the study (maintenance period) for a minimum of 6 months. All randomised patients who discontinue study drug for any reason other than death will be followed for the entire duration of the study; patients who undergo renal transplantation or dialysis will be followed for vital status only.

Outcomes <u>Efficacy</u>:

- The primary outcome measure will consist of rate of decline in renal function as measured by glomerular filtration rate (creatinine clearance).
- The secondary outcome measures to be evaluated are development of diabetic retinopathy, diabetic peripheral neuropathy, and diabetic autonomic neuropathy.

<u>Safety:</u>

- Safety parameters evaluated will be adverse events and clinical laboratory

abnormalities which will be assessed at time points by medical history, physical examination, and laboratory analyses and compared between groups.

Statistical considerations: The sample size estimate for this trial is determined for the primary hypothesis that the projected rate of decay of creatinine clearance (1mL.min-1) in NIDDM patients with diabetic nephropathy (creatinine clearance <90 mL.min-1) will be reduced by treating with triene. The study is powered to detect (80%) a 6mL.min-1 change in creatinine clearance over 6 months with four 2-monthly readings (i.e. 0, 2, 4, & 6 months) assuming a 10% rate of loss to follow-up at the 5% significance level.

CONCLUSION:

16. The **efficacy** of triene as a treatment of microvascular complications in patients with NIDDM will be confirmed.
17. The **safety** of long-term administration of triene in patients with poor blood glucose control and diabetic nephropathy due to NIDDM will be confirmed.
18. It also provides a means to determine the clinical usefulness of alternative *fructosamine oxidase* inhibitors such as the copper chelating compounds *D*-penicillamine, sar, and diamsar (ie; triene could be used in place of placebo in ensuing clinical trials).

WHAT IS CLAIMED IS:

1. A method of treating a mammalian patient (human or otherwise) predisposed to and/or suffering from diabetes mellitus with a view to minimising the consequences of macrovascular and microvascular damage to the patient (eg. accelerated atherosclerosis, blindness, renal failure, neuropathy, etc.) which comprises, in addition to any treatment in order to control blood glucose levels, at least periodically inhibiting or antagonising fructosamine oxidase enzyme activity in the patient.

2. A method of claim 1 wherein said inhibition or antagonism occurs as a result of administration or self-administration of at least one fructosamine oxidase reaction product inhibitor or antagonist.

3. A method of claim 2 wherein any such inhibitor or antagonist is selected from the groups

(i) copper chelating agents

(ii) substrate analogue

(iii) hydrazine compound.

4. A method of claim 2 wherein said inhibitor or antagonist is taken orally.

5. A method of claim 2 wherein said inhibitor or antagonist is taken orally as part of a regime, whether totally oral or not, which also involves the control of blood glucose levels.

6. A pharmaceutical composition suitable for use in a method of treating a mammalian patient predisposed to and/or suffering from diabetes mellitus with a view to minimising macrovascular and microvascular damage, said composition comprising a fructosamine oxidase inhibitor or antagonist in conjunction with a suitable carrier therefor.

7. A pharmaceutical composition for reducing macrovascular and microvascular damage in a mammalian patient (including a human) suffering from diabetes mellitus, said composition comprising a fructosamine oxidase inhibitor or antagonist and suitable carrier therefor.

8. The use of a fructosamine oxidase inhibitor or antagonist in the manufacture of a pharmaceutical composition comprising the fructosamine oxidase inhibitor or

antagonist and a suitable pharmaceutical carrier therefor and which composition is useful in treating a mammalian patient (human or otherwise) which or who is suffering from diabetes mellitus to reduce macrovascular and microvascular damage.

9. In combination, a pharmaceutical composition of claims 6 or 7 and a pharmaceutical composition effective at lowering blood glucose levels.

10. A method of treating a mammalian patient (human or otherwise) predisposed to and/or suffering from diabetes mellitus, which includes inhibiting or antagonising fructosamine oxidase enzyme activity in the patient with an agent or agents not contraindicated for the patient.

11. A method of claim 10 wherein said agent(s) is or are copper chelating compound(s) administered or self administered to the patient.

12. A method of claim 11 wherein said copper-chelating compound is triethylenetetramine dihydrochloride (triene), ethylenediamine tetraacetic acid, *o*-phenanthroline, or histidine.

13. A method of claim 10 wherein said agent(s) is or are substrate analogue compound(s) administered or self administered to the patient having an amino acid or peptide moiety with a blocked N-terminal amine group.

14. A method of claim 13 wherein said substrate analogue composition is *N*-acetylcysteine, captopril, or enalapril.

15. A method of claim 10 wherein said agent(s) is or are hydrazine compound(s) administered or self administered to the patient ie: a compound having a **-NHNH$_2$** moiety.

16. A method of claim 15 wherein said hydrazine compound is diaminoguanidine, hydralazine, or carbidopa.

17. A dosage unit or pharmaceutical composition for a patient useful in a method of claim 10 comprising (preferably in effective fructosamine oxidase reaction product inhibiting or antagonising amounts - separately or collectively) of a compound (or compounds) being a substrate analogue or a hydrazine compound having an **-NHNH$_2$** moiety, or both.

18. A dosage unit or composition of claim 17 which is in an oral dosage form optionally with carriers, excipients or, indeed, even other active agents (e.g. means

to lower blood glucose levels).

19. A regime or dosage unit or pharmaceutical composition for a diabetic or suspected diabetic patient of captopril [whether effective or intended to be effective in controlling blood pressure of the diabetic patient (at least in part) or not] providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

20. A regime or dosage unit or pharmaceutical composition for a diabetic patient or suspected diabetic patient of

(i) a hydrazine compound and

(ii) at lease one other fructosamine oxidase inhibitor and/or antagonist, the mix of (i) and (ii) providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

21. A regime or dosage unit or pharmaceutical composition for a diabetic patient or suspected diabetic patient of

(i) acetylcysteine and

(ii) at lease one other fructosamine oxidase inhibitor and/or antagonist, the mix of (i) and (ii) providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

22. A regime or dosage unit or pharmaceutical composition for a diabetic patient or suspected diabetic patient of

(i) hydralazine and

(ii) at lease one other fructosamine oxidase inhibitor and/or antagonist, the mix of (i) and (ii) providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

23. A method of treating a mammalian patient (human or otherwise) predisposed to and/or suffering from diabetes mellitus which includes inhibiting and/or antagonising fructosamine oxidase enzyme activity in the patient with either:

(a) acetycysteine and hydralazine, or

(b) acetylcysteine and carbidopa.

24. A regime or dosage unit or pharmaceutical composition for a diabetic or suspected diabetic patient which includes either:

(i) acetylcysteine and hydralazine, or

(ii) acetylcysteine and carbidopa.

25. The use of co-administration or serial administration of acetylcysteine and hydralazine for the purpose of reducing micro-vascular damage in a mammal.

26. The use of claim 25 wherein said mammal is diabetic.

27. The use of co-administration or serial administration of acetylcysteine and carbidopa for the purpose of reducing microvascular damage in a mammal.

28. The use of claim 27 wherein said mammal is diabetic.

29. A regime or dosage unit or pharmaceutical composition for a diabetic or suspected diabetic patient of the copper chelator, triene, providing for the patient a sufficient fructosamine oxidase inhibiting and/or antagonising effect to reduce macrovascular and microvascular damage.

30. A method of treating and/or reducing the likelihood of diabetic cataract in a mammal which comprises at least periodically inhibiting and/or antagonising fructosamine oxidase enzyme activity in the mammal.

31. A method of claim 30 which involves the administration or self administration of effective amounts of triethylenetetramine dihydrochloride (triene).

32. A method of treating and/or reducing the likelihood of diabetic cardiomyopathy in a mammal which comprises at least periodically inhibiting and/or antagonising fructosamine oxidase enzyme activity in the mammal.

33. A method of claim 32 which involves the taking or administration of effective amounts of triethylenetetramine dihydrochloride (triene).

34. A method of claim 1, claim 10, claim 30 or claim 32 herein triethylenetetramine dihydrochloride (triene) is administered and/or self administered in concert with another fructosamine inhibitor and/or antagonist or other fructosamine oxidase enzyme inhibitors and/or antagonists.

35. A method of claim 34 wherein said another inhibitor and/or antagonist or other inhibitors and/or antagonists are administered or self administered to elicit a

pharmacological effect for another indication yet together with the effect of the triene is or are effective for treating or ameliorating macrovascular and microvascular damage of such a patient or mammal.

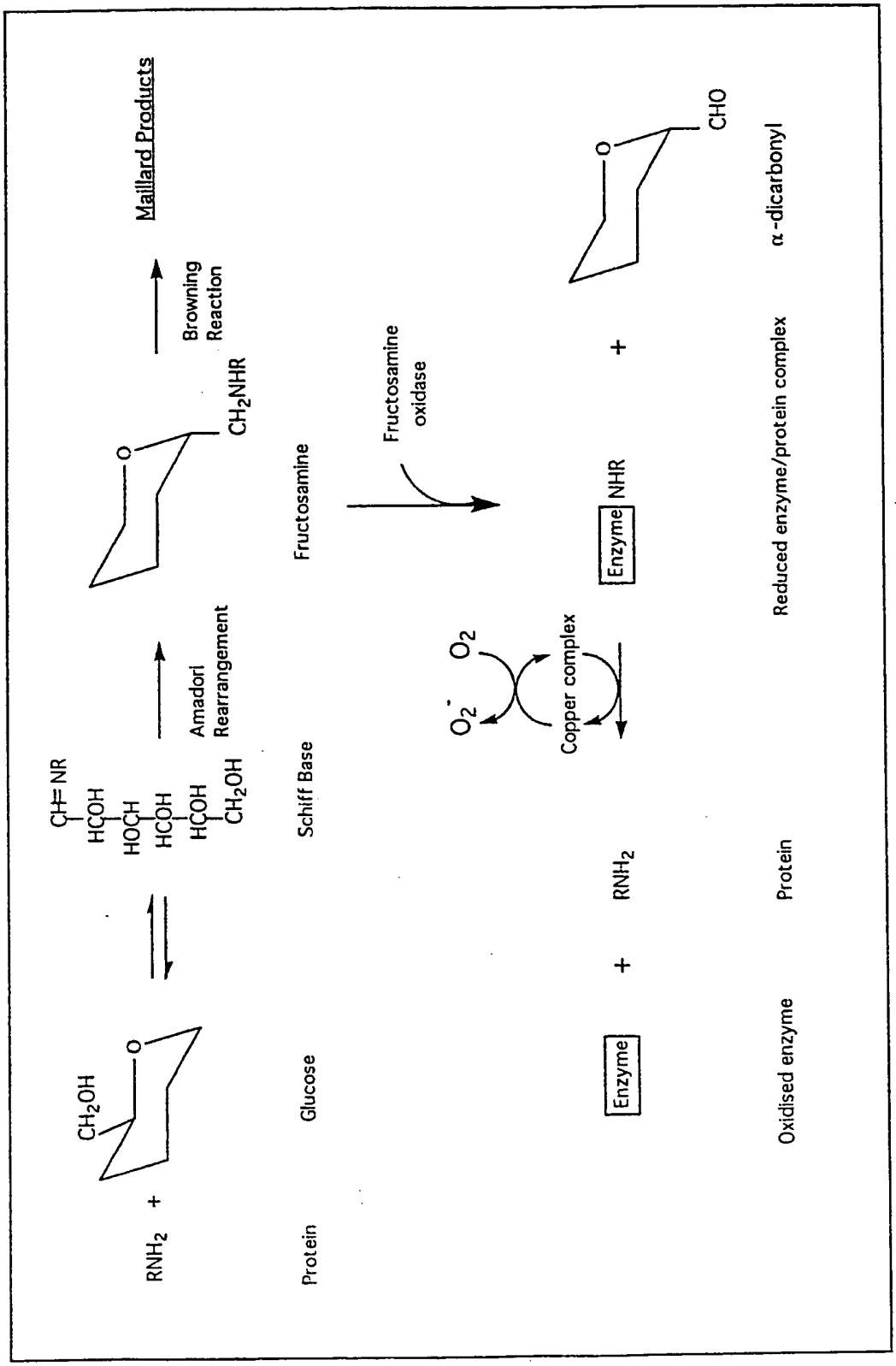
Maillard Products
Browning Reaction
Amadori Rearrangement
Fructosamine oxidase
Copper complex
Schiff Base
Fructosamine
Glucose
Protein
Enzyme
Oxidised enzyme
Reduced enzyme/protein complex
α -dicarbonyl

FIGURE 1.

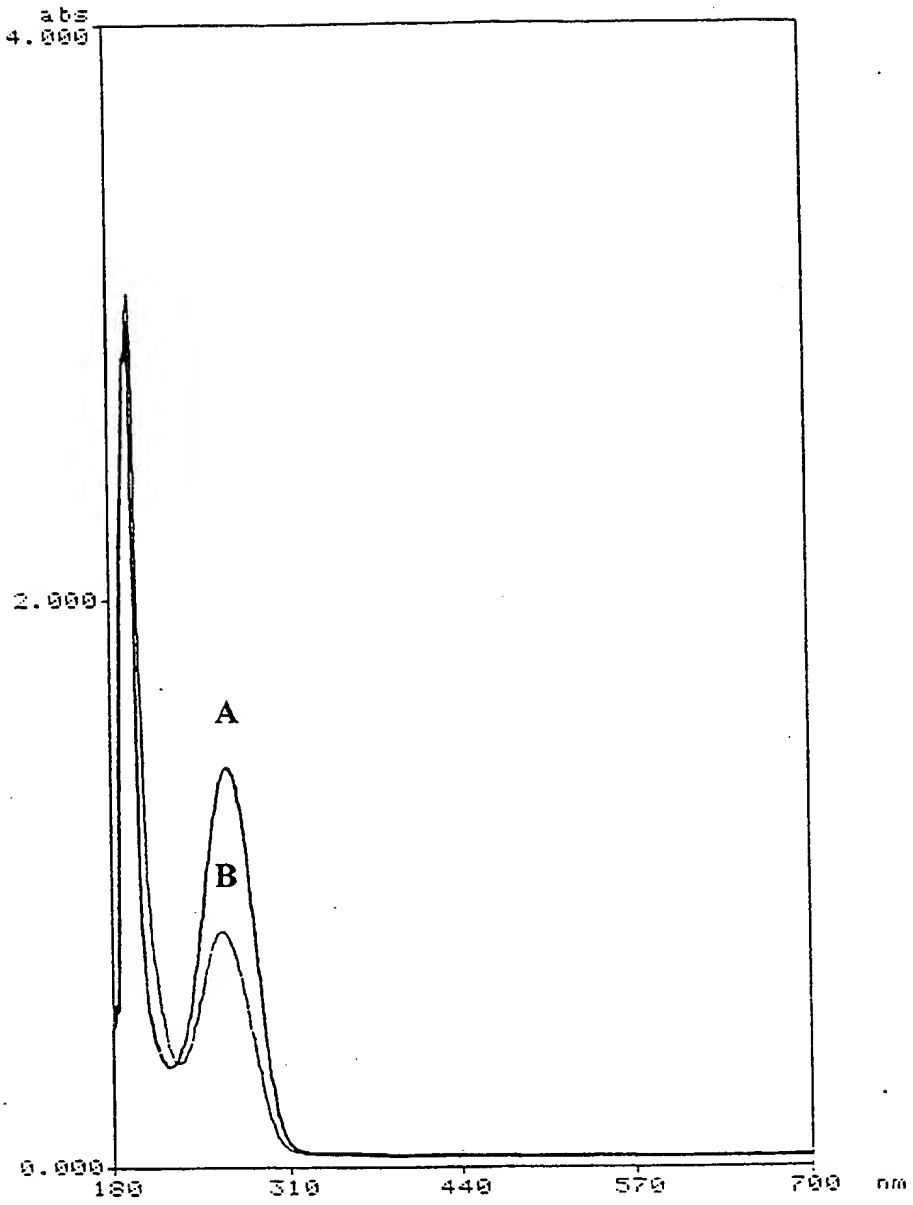

FIGURE 2

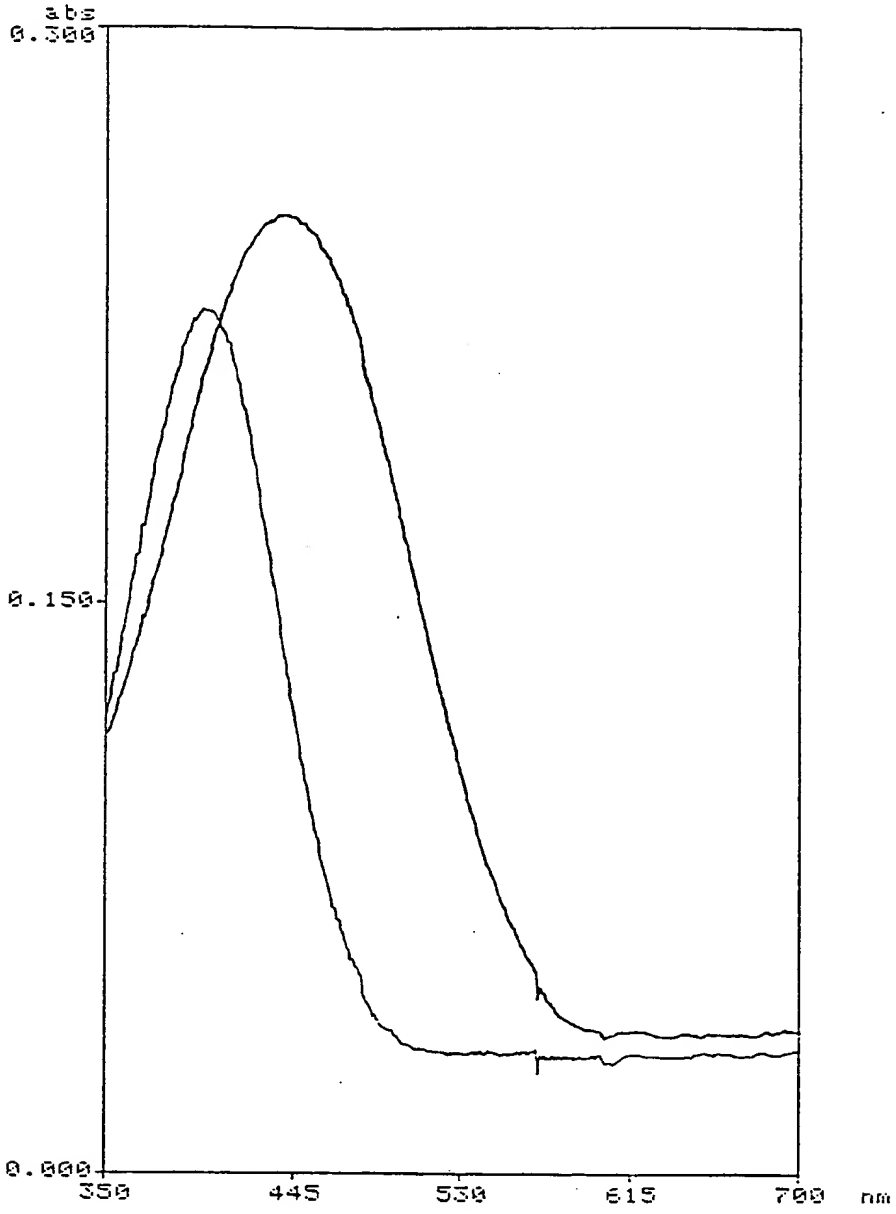

FIGURE 3

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Figure 4

- **Survival (%)**
- **Treatment groups**
  - non-diabetic (1)
  - diabetic control (2)
  - hydralazine (3)
  - EDTA (4)
  - acetylcysteine & hydralazine (5)
  - acetylcysteine (6)

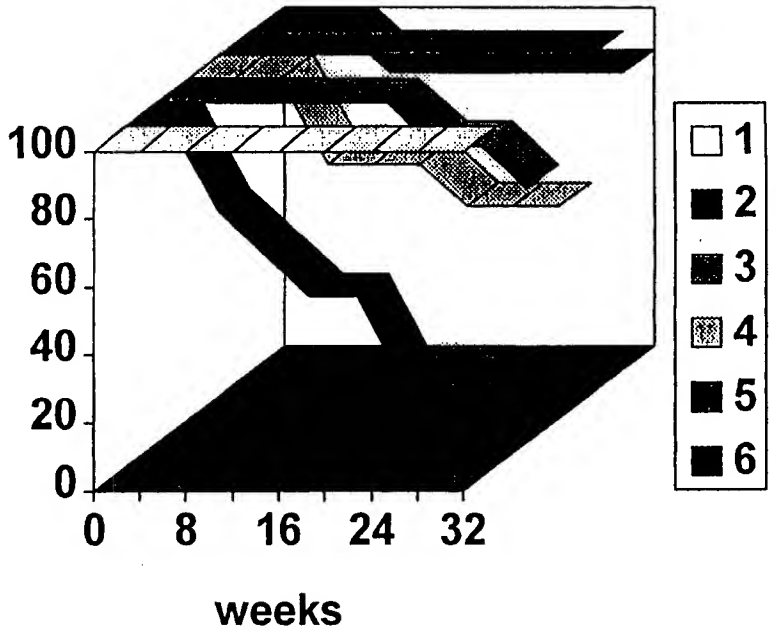
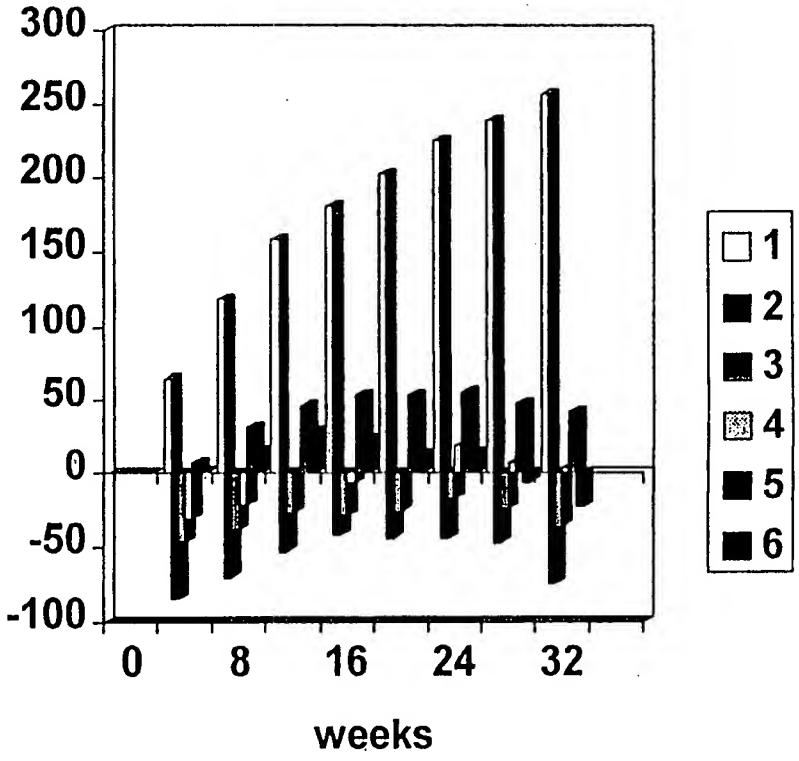


Figure 5

- **weight change**
  - group mean (gms)
- **Treatment groups**
  - non-diabetic (1)
  - diabetic control (2)
  - hydralazine (3)
  - EDTA (4)
  - acetylcysteine & hydralazine (5)
  - acetylcysteine (6)



INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ99/00161

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61K 031/15, A61K 031/155, A61K 031/16, A61K 031/175, A61K 031/195, A61K 031/275, A61K 031/40, A61K 031/44, A61K 031/50, A61K 031/655

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: A61K, SEARCH TERMS AS BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Derwent, C.A. (STN):- diabet: AND (phenylhydrazine OR lisinopril OR enalapril OR captopril OR acetylpenicillamine OR desferrioxamine OR EDTA OR aminoguanidine OR semicarbazide OR benseramide OR oxalic()dihydrazide OR hydralazine OR potassium()cyanide OR triene OR o-phenanthroline OR histidine OR sodium()azide)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99/39712A (COCENSYS, INC.) 12 August 1999 See whole document	1 - 10, 15, 17 - 18
X	WO 87/05505 A (EUROSIUM LABAROTORIES INC.) 24 September 1987 See whole document	1 - 10, 11 - 12
X	DE 3217071 A (GRÖNING, RÜDIGER, Dr.) 10 November 1983 See whole document	1 - 10, 15 - 18

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

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Date of the actual completion of the international search	Date of mailing of the international search report
07 December 1999	21 DEC 1999

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